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Nuclear localisation of calreticulin in vivo is enhanced by its interaction with glucocorticoid receptors

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Abstract The multi-functional protein calreticulin (CRT) is normally found within the lumen of the endoplasmic reticulum (ER). However, some of its proposed functions require it to be located within the nucleus, where its presence is contentious. We have investigated this in live COS7, HeLa and LM(TK$^-$) cells using green fluorescent protein (GFP)-fusion proteins. GFP-CRT, and GFP, with an ER signal peptide and a KDEL sequence (ER-GFP), were localised to the ER. In addition, GFP-CRT was located in the nucleus of all the cell types at low levels. The higher levels of nuclear fluorescence in LM(TK$^-$) and HeLa cells suggested that glucocorticoid receptors might enhance nuclear localisation of calreticulin. Dexamethasone treatment of LM(TK$^-$) cells doubled the amount of nuclear GFP-CRT, but did not affect the localisation of a GFP-CRT fusion in which the glucocorticoid receptor-binding N-domain of calreticulin had been deleted. Thus, despite ER targeting and retention signals, calreticulin is also located within the nucleus where its presence increases due to its interaction with glucocorticoid receptors.

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Key words: Endoplasmic reticulum; Calreticulin; GFP (green fluorescent protein); Glucocorticoid receptor; Confocal microscopy; Nucleus

1. Introduction

In addition to its apparent major role as a calcium storage protein within the lumen of the ER, calreticulin has been proposed to have functions that necessitate its presence at cellular locations other than the ER [1]. These include the nucleus, where it regulates steroid-inducible gene expression [2–4], and the cytosol, where it is involved in the control of cell adhesion via interaction with the α subunits of integrins [5]. The presence of calreticulin in the nucleus has been demonstrated by both immunocytochemistry [6,7], and sub-cellular fractionation [8]. However, due to staining and fixation artefacts inherent in the immunocytochemical approaches [9], the possibility of contamination in sub-cellular fractionation, and the indirect nature of the functional studies, the presence of calreticulin in the nucleus remains contentious. Indeed, it has been suggested that the constitutive presence of nuclear calreticulin may be artefactual [10]. Furthermore, although there is a putative nuclear localisation signal (PPKKIKDPD) in calreticulin [11], it remains unclear how a protein containing an N-terminal ER-targeting signal and a C-terminal KDEL salvage sequence [12] escapes ER retention and translocates to the nucleus. One possibility is that a nuclear-specific isoform exists, but although a number of calreticulin isoforms have been found in bovine tissues, all have both the ER targeting and retention signals [13].

We have addressed these problems by engineering fusions of calreticulin and GFP [14] that have allowed us to investigate directly calreticulin distribution in live cells. The data show that, despite ER targeting and retention signals, calreticulin is also located within the nucleus. Furthermore, its presence within the organelle is enhanced as a result of the protein's interaction with glucocorticoid receptors.

2. Materials and methods

2.1. Materials

Plasticware and tissue culture reagents were from GibcoBRL (Paisley, UK). Cell lines were obtained from the European Tissue Culture Collection (Porton Down, UK). Restriction and modifying enzymes were purchased from Promega (Southampton, UK) and Bioline (London, UK). General laboratory chemicals were from Sigma (Poole, UK), or Fisons (Loughborough, UK). The anti-GFP antibody was obtained from Clontech (Palo Alto, USA), and the Cy3 goat anti-rabbit IgG came from Zymed (San Francisco, USA).

2.2. Engineering of GFP fusions and cloning

A cDNA containing the whole of the calreticulin protein-coding sequence was generated from HL60 mRNA by reverse transcription-polymerase chain reaction (RT-PCR). Primers were derived from the published sequence [15] (figures in parentheses refer to the sequence positions given therein):

Primer 1: calreticulin forward primer (−56)
5′GCCGCTGCCGGGAGGGTGGTTTT3′

Primer 2: calreticulin reverse primer (+1432)
5′AAGGGCGGGGAGGGGGTGGGGG3′

Both the wild-type (wt) GFP [14] and its S65T variant [16], which forms its fluorescent chromophore more rapidly at 37°C, and has an excitation wavelength more suitable for FITC filter sets, were obtained from Clontech, Palo Alto, USA. These were used in a two-stage PCR strategy [17] to generate GFP-CRT chimeric cDNA constructs. The following constructs were engineered using the primers indicated:

ER-GFP: CRT ER signal peptide-GFP(wt/S65T)+KDEL

Primer 3: *part of calreticulin signal peptide*+GFP forward primer
5′*gtgccgctgctgctcggctcctcggcctggccgtcgcca*GTAAAGAGAAGAACTT3′

Primer 4: GFP reverse primer+*kdel*
5′*ttacagctcatcctt*TTTGTATAGTTCATCCAT3′

GFP-CRT: CRT ER signal peptide/GFP/CRT(no signal peptide+KDEL)

Primer 3: *part of calreticulin signal peptide*+GFP forward primer (as above)

Primer 5: reverse primer for GFP-*calreticulin* fusion site
5′*gtagacggcgggctc*TTTGTATAGTTCATC3′

Primer 6: forward primer for GFP-*calreticulin* fusion site
5′GATGAACTATACAAA*gagcccgccgtctac*3′

Primer 2: calreticulin reverse primer (+1432) (as above)

Primer 7: to add the N-terminus of calreticulin signal sequence to both constructs:
5′CCCGCCATGCTGCTGCTATCCGTGCCGCTGCTGCTC3′

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A fusion was also generated in which the N-domain (amino acids 1–180) of the calreticulin protein [18] was deleted:

GFP-CRT(ΔN):	**CRT ER signal peptide/GFP/CRTΔN (no signal peptide+KDEL)**
Primer 8:	forward primer for GFP and *calreticulin(ΔN)* fusion. $^{5'}$*ttccaaggagccgga*TTTGTATAGTTCATC$^{3'}$
Primer 9:	reverse primer for GFP and *calreticulin(ΔN)* fusion. $^{5'}$GATGAACTATACAAA*tccggctccttggaa*$^{3'}$

In all cases, the PCR generated products were cloned into pCR3, a TA cloning vector with a CMV immediate-early promoter (Invitrogen, The Netherlands).

2.3. Cell culture and transfection methods

Cell lines were grown in Dulbecco's Modified Eagle Medium (DMEM) without phenol red, supplemented with 10% foetal calf serum, 2 mM L-glutamine, 100 μg/ml penicillin, 100 μg/ml streptomycin and 100 μg/ml amphotericin B. Cells were transfected using the calcium phosphate method. Medium was changed 24 h post transfection and 24 h later cells were re-seeded at 2×10^4 for COS7 cells, and 6×10^4 for both HeLa and LM(TK$^-$) cells in the centre of a 22×22 mm glass cover slip.

In experiments where dexamethasone was added, cells were washed in serum-free medium and incubated with 1 μM dexamethasone for 30 min. The relatively short incubation time has been shown to be sufficient for maximal nuclear translocation of the glucocorticoid receptor in response to dexamethasone [19,20].

2.4. Immunocytochemistry

Cells were fixed with 2% paraformaldehyde, 0.1% glutaraldehyde for 15 min at room temperature. After washing with phosphate-buffered saline (PBS) pH 7.4, the cells were permeabilised with 0.1% Triton X-100/0.1 M lysine. Following incubation with 10% (v/v) pre-immune goat serum/PBS for 1 h, cells were incubated overnight at 4°C with a rabbit anti-GFP antibody. Detection was by a Cy 3 conjugated secondary antibody. The cover slips were mounted with Vectashield (Vector Laboratories, Peterborough, UK)

2.5. Observation and quantification of GFP fluorescence in live cells

Cells were observed using excitation (488 nm), and emission (515 nm) wavelengths for FITC, using a Leica TCS 4d confocal laser scanning microscope. The objective, lens, pinhole diameter, and sensitivity of detector were constant for all sections. A series of sections through each fluorescent cell was performed, with the section through the centre of the nucleus chosen for analysis. Photometric analysis of digitised confocal sections was performed using Photek image analysis software (Photek, St. Leonards-on-Sea, UK). In order to determine the degree of nuclear fluorescence, and account for differences in cell size and relative fluorescence intensity, areas corresponding to the nucleus and the whole cell were encircled, and the ratio of nuclear to whole cell fluorescence determined [21].

3. Results

3.1. The ER-targeted GFP S65T variant fluoresces in the ER lumen.

Since calreticulin is primarily an ER lumenal protein, we first determined whether GFP fluorescence could be detected within the ER lumen of COS7 cells, employing a targeting strategy involving the use of the calreticulin N-terminal signal peptide and the C-terminal KDEL salvage sequence [22]. Immunolocalisation with an anti-GFP antibody confirmed that both wt and S65T ER-targeted GFP constructs were expressed and localised in the ER. However, GFP fluorescence was only observed with the S65T variant (data not shown). No nuclear fluorescence was visible with the S65T ER-GFP fusion (Fig. 1a), and photometric analysis showed that the ratio of nuclear to whole cell fluorescence was less than 0.001.

3.2. GFP-CRT localises predominantly to the ER, but is also found in the nucleus

Having demonstrated that the S65T variant fluoresces within the ER lumen, it was used to generate a fusion (GFP-CRT) with calreticulin by inserting GFP between the calreticulin N-terminal signal peptide and the remainder of the protein. In contrast to ER-GFP (Fig. 1a), fluorescence from the GFP-CRT fusion was visible in the nucleus of all the cell types (Fig. 1b,c, and Table 1). Photometric analysis showed that the ratio of nuclear to whole cell fluorescence in COS7 cells, which displayed the least amount of GFP-CRT nuclear fluorescence, was at least 4 times higher than the level observed for ER-GFP.

In order to confirm that the presence of the GFP-calreticulin fusion within the nucleus was not due to mis-targeting as a result of its overexpression, we performed western blotting of cell lysates of GFP-CRT transfected cells using an anti-calreticulin antibody that we raised against the whole recombinant protein. In addition to detecting endogenous calreticulin, there was a single band of the expected size for the correctly processed GFP-CRT fusion, i.e. the signal peptide had been efficiently cleaved (data not shown). A previous study has shown that where calreticulin was overexpressed 50–100-fold per cell its distribution was unaffected [23]. Thus, we infer that the nuclear localisation of the GFP-CRT fusion does not arise from its mis-targeting due to saturation of the ER translocation machinery, but occurs as a result of trafficking due to sequence(s) present within the calreticulin moiety of the chimera. However, an alternative explanation for our observations was suggested by a recent report showing that invaginations of the ER extend into, and through the nucleus [24], raising the possibility that the GFP-CRT fusion protein is present in such invaginations and not the nucleus per se. To exclude this possibility, confocal sections of the transfected cells were taken across all planes showing that the nuclear calreticulin detected was within the nucleoplasm and not invaginating ER cisternae.

3.3. Nuclear localisation of calreticulin is enhanced by the interaction of its N-domain with glucocorticoid receptors

Photometric analysis showed that the degree of GFP-CRT nuclear fluorescence varied considerably between the cell types, with 10-fold and 6-fold less nuclear GFP-CRT in COS7 cells than in LM(TK$^-$) cells and HeLa cells, respectively (Table 1). Unlike COS7 cells, both HeLa cells and LM(TK$^-$) cells possess glucocorticoid receptors with which calreticulin interacts [2,3]. This suggested that the greater level of nuclear GFP-CRT in these cells, compared to COS7 cells, might be due to this interaction, resulting in its co-translocation to the nucleus with glucocorticoid receptors. Therefore, we determined whether GFP-CRT nuclear localisation in HeLa cells and LM(TK$^-$) cells was affected by treatment with the synthetic glucocorticoid dexamethasone which activates the glucocorticoid receptors causing them to translocate to the nucleus [25]. This treatment resulted in a 2-fold increase in nuclear fluorescence of GFP-CRT transfected in LM(TK$^-$) cells, but, in contrast, had no effect in similarly treated HeLa cells or COS7 cells (Table 1). The distribution of the ER-GFP construct was unaffected by dexamethasone. These observations are in accordance with previous reports that only in certain glucocorticoid receptor containing cells, including LM(TK$^-$) cells, but not HeLa cells, can dexamethasone-

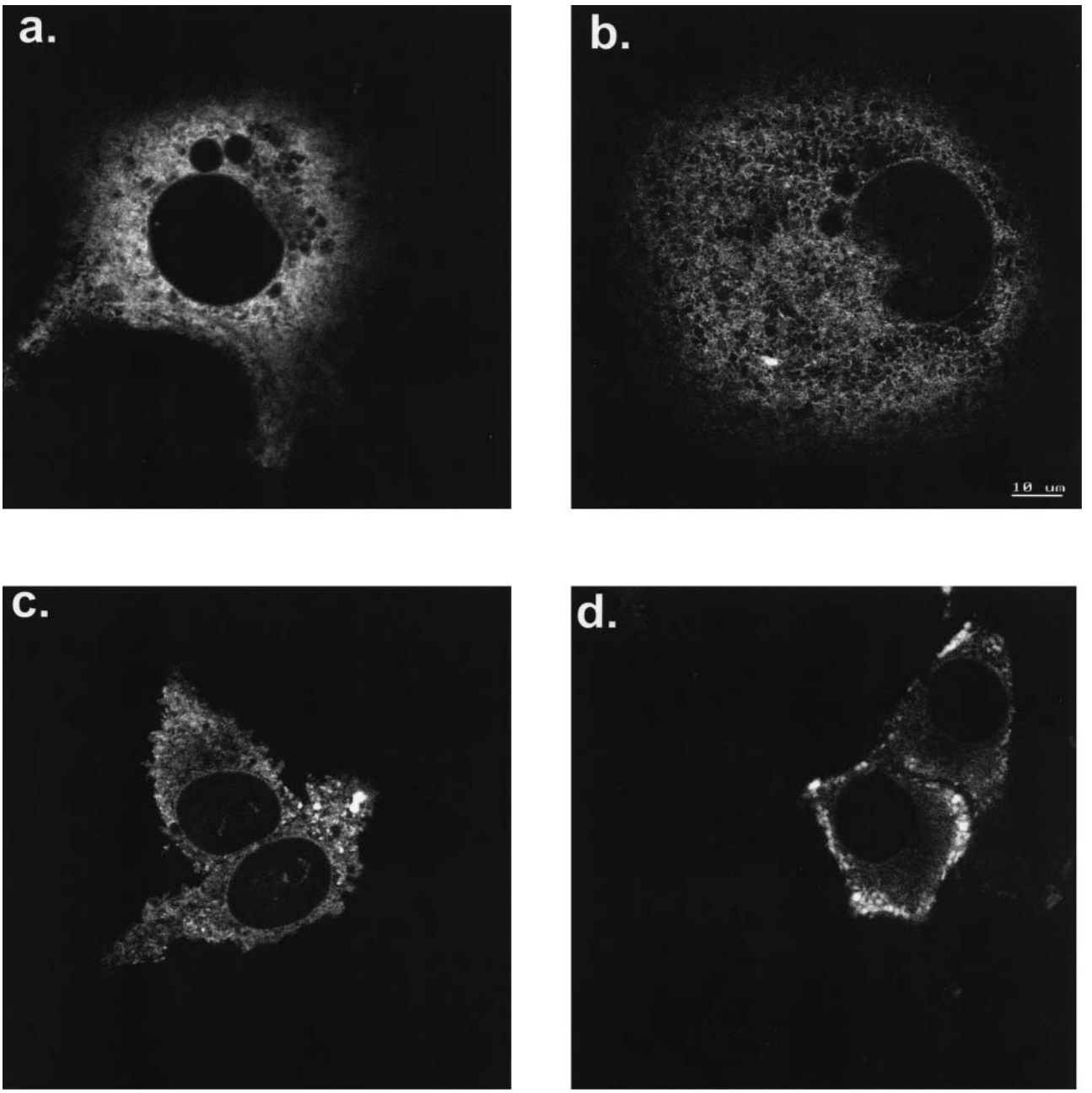

Fig. 1. Fluorescence of ER-GFP and GFP-CRT fusions in COS7 and LM(TK$^-$) cells. Typical confocal sections of live transfected cells observed under ×1000 initial magnification. GFP fluorescence was detected using FITC excitation and emission wavelengths. None of the cells illustrated had been treated with dexamethasone. (a) ER-GFP fluorescence in COS7 cells showing a typical ER network with no fluorescence visible in the nucleus. (b) GFP-CRT fluorescence in COS7 cells. In addition to the expected ER distribution, there is also fluorescence within the nucleus unlike the ER-GFP fusion shown in panel (a). (c) GFP-CRT fluorescence in LM(TK$^-$) cells. As with COS7 cells, there is again fluorescence within the nucleus, calculated by photometry to be 10-fold greater than that in COS7 cells (see Table 1). (d) GFP-CRT(ΔN) fluorescence in LM(TK$^-$) cells. Fluorescence is again visible within the nucleus, but is 5.7-fold lower than that for the full-length GFP-CRT fusion, demonstrating the role of the calreticulin N-domain in its nuclear localisation.

stimulated nuclear translocation of endogenous glucocorticoid receptors be detected [20,25,26]. In order to substantiate further the involvement of glucocorticoid receptors in the nuclear localisation of calreticulin, we performed similar experiments in LM(TK$^-$) cells utilising a GFP-CRT construct in which the glucocorticoid receptor-binding N-domain of the calreticulin protein had been deleted. Again, this showed essentially an ER distribution, but with fluorescence also present in the nucleus. However, this nuclear fluorescence was 5.7-fold lower than that of the full-length GFP-CRT construct, and was unaffected by dexamethasone treatment (Fig. 1c,d, and Table 1).

Thus, we conclude that, despite ER targeting and retention signals, calreticulin is found within the nucleus. Furthermore, the degree of nuclear localisation of calreticulin appears to be dependent on the presence of glucocorticoid receptors inferring that the protein is able to enter the nucleus in association with such receptors.

Table 1
Quantitative analysis of nuclear fluorescence from the GFP-CRT fusions, and the effect of dexamethasone treatment

Construct	Cell type	Ratio of nuclear to total cell fluorescence	
		−dexamethasone	+dexamethasone
GFP-CRT	COS7	0.004 (±0.001)	0.005 (±0.001)
	HeLa	0.024 (±0.005)	0.025 (±0.004)
	LM(TK$^-$)	0.040 (±0.010)	0.081 (±0.013)[a]
GFP-CRT(ΔN)	LM(TK$^-$)	0.007 (±0.004)	0.006 (±0.003)

This was performed as described in Section 2. At least 15 cells were chosen at random in three separate experiments. The figures represent the means of the calculated ratios of nuclear to total cell fluorescence ± standard deviation.
[a] $p < 0.05$, Student's t-test (two-tailed, assuming unequal variance).

4. Discussion

Several of the functions ascribed to calreticulin necessitate that it is located within the nucleus where its presence has been contentious. Employing GFP as an in vivo reporter of calreticulin cellular distribution, we have demonstrated conclusively that calreticulin is found within the nucleus in addition to its expected ER localisation. It has been suggested that there may be a nuclear-specific isoform of the protein, but, like the endogenous protein, our fusions possess an N-terminal ER targeting sequence and a C-terminal retention signal which may argue against the existence of such an isoform. Our data showed low levels of calreticulin within the nucleus of all the cell types we studied, but there was much less in COS7 cells than either LM(TK$^-$) or HeLa cells. It may be that calreticulin is totally absent from the nucleus in some types of cells, although it is possible that its levels are too low to be detected by antibody-dependent techniques.

A number of crucial questions arise concerning the mechanism and route by which ER-targeted calreticulin locates to the nucleus. The protein has a putative nuclear localisation signal (NLS) in its P-domain [1]. It has been shown that injection of FITC-labelled calreticulin into the cytosol of fibroblasts leads to transient nuclear localisation of the protein [27], implying a route into the nucleus from the cytosol, where calreticulin has been detected [10]. Our data provide strong evidence that calreticulin can also locate to the nucleus from the cytosol by virtue of its interaction with glucocorticoid receptors. First, there were higher levels of nuclear calreticulin in LM(TK$^-$) and HeLa cells, which contain glucocorticoid receptors, than in COS7 cells which lack them. Secondly, the deletion of the glucocorticoid receptor-binding N-domain of calreticulin from our fusion reduced its levels within the nucleus of LM(TK$^-$) cells to those seen in COS7 cells and, unlike the wild-type calreticulin fusion, these did not increase in response to dexamethasone treatment. Ligand binding causes dissociation of glucocorticoid receptors from their cytosolic multi-protein complexes resulting in their translocation to the nucleus [28]. The effect of ligand stimulation upon the localisation of endogenous glucocorticoid receptors has been studied in a variety of cell types showing that discernible nuclear translocation is detected only in certain cell types, including LM(TK$^-$) cells, but not others, such as HeLa [20,25,26]. These observations might explain why dexamethasone had no effect upon nuclear calreticulin in HeLa cells, but appeared to increase its levels in LM(TK$^-$) cells. The appearance of calreticulin in the nucleus prior to dexamethasone stimulation might be attributable to the presence of glucocorticoid receptors within the nucleus of unstimulated cells [21,26], as well as the effect of the NLS which presumably accounts for the presence of calreticulin in the nucleus of COS7 cells. We are currently mutating this signal and other regions of the calreticulin protein to investigate their contribution to its nuclear localisation. It also remains to be resolved how calreticulin locates to the cytosol from the ER.

The conclusive demonstration that calreticulin is in the nucleus is important for the roles that have been attributed to the protein. It can modulate steroid-inducible gene expression [2,3] and it might also contribute to the control of nuclear calcium signalling. Furthermore, it has recently been shown that irradiation of radioresistant squamous carcinoma epithelial cells caused translocation of calreticulin into the nucleus [8]. Thus, it may be that calreticulin within the nucleus is involved in controlling important cellular processes such as differentiation, cell death, and apoptosis.

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